Discovery and Cocrystal Structure of Benzodiazepinedione HDM2 Antagonists That Activate p53 in Cells

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Received October 26, 2004

Abstract: HDM2 binds to an α-helical transactivation domain of p53, inhibiting its tumor suppressive functions. A miniaturized thermal denaturation assay was used to screen chemical libraries, resulting in the discovery of a novel series of benzodiazepinedione antagonists of the HDM2−p53 interaction. The X-ray crystal structure of improved antagonists bound to HDM2 reveals their α-helix mimetic properties. These optimized molecules increase the transcription of p53 target genes and decrease proliferation of tumor cells expressing wild-type p53.

The p53 tumor suppressor plays a key role in regulating cell cycle progression, as evidenced by the high frequency of p53 mutations in neoplasia.[1] p53 is a transcription factor that regulates genes that can induce either cell cycle arrest or apoptosis. In human cells, p53 activity is normally modulated by HDM2, the homologue of murine MDM2. HDM2 binds to and blocks the p53 transactivation domain, preventing the transcription of p53 target genes. Both p53 and HDM2 are covalently modified in response to cell stress, resulting in their dissociation. This disruption of the HDM2−p53 interaction can be mimicked by neutralizing antibodies,[2] p53 peptides,[3] or protein fusions[4,5] and leads to p53 stabilization and control of proliferation. Additionally, antisense HDM2 oligonucleotides increase both p53 levels and activity by reducing HDM2 protein levels.[6] The crystal structure of the HDM2 N-terminal domain bound to a p53 peptide[7] revealed that this association is driven largely by three amino acid side chains in close proximity to each other on one face of a helix, raising the expectation that a small molecule antagonist might be found that could mimic this interaction and accomplish similar disruption and cellular sequelae. Such a molecule would be expected to have therapeutic potential for the estimated 7% of human tumors that overexpress HDM2,[8] as well as the 50% of tumors that express wild type (wt) p53.

Low-molecular weight antagonists of HDM2 have been reported, but many of these molecules have had low potency and limited characterization of their cellular activity.[9−11] Most recently, a report describing compounds with good potency and in vivo activity also provided structural data demonstrating that these compounds bind HDM2 in the same pocket as the p53 helix.[12] We describe here the discovery and characterization of small molecules of a different chemotype that bind to HDM2 with high affinity resulting in functional agonism of p53 in cells.

Compound libraries designed using Directed Diversity software[13−15] were synthesized using standard combinatorial chemistry methods. More than 338 000 compounds were screened for HDM2 binding using a miniaturized affinity-based screening assay, named ThermoFluor.[16] The ThermoFluor instrument uses fluorescent dyes to monitor protein unfolding as a function of temperature, allowing the detection of compound binding to target proteins by measuring the resultant increase in thermal stability. This is quantified as the change in midpoint transition temperature (ΔT_m) in the presence of the compound at a single concentration.[16] The protein used in these experiments, glycine-HDM2 residues 17−125, comprises a minimal N-terminal p53 binding domain.[7] That the ThermoFluor assay is a sensitive measure of binding affinity for HDM2 is verified by the shift in T_m on addition of peptides known to bind to HDM2,[17,18] with higher affinity peptides generating larger shifts (Figure S1).

On the basis of a statistical analysis of ThermoFluor-generated melting curves, 1216 compounds were selected for further characterization. Included in this group were 116 compounds (ΔT_m 1.0− 4.9 °C) from a benzodiazepinedione (BDP) library, an example of which is shown in Figure 1A. The BDPs have two chiral centers and were screened as mixtures of four stereoisomers. The selected compounds were tested using a fluorescent peptide displacement (FP) assay designed to detect specific inhibitors of the p53-HDM2 interaction, and the most active BDPs were used as guides for synthetic elaboration.[19] The more active pair of enantiomers from an optimized analogue were separated using chiral chromatography, and their divergent affinities confirm the stereospecificity of binding (Figure 1B).

To understand the molecular details of BDP binding by HDM2, we determined crystal structures of HDM2 with various peptides and small molecules occupying the p53 binding pocket. We solved the structures of two in a series of optimized peptides reported to have affinities that decreased with peptide length[18] to determine if the HDM2 binding site changed with inhibitor

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10.1021/jm049137g CCC: $30.25
Published on Web 02/01/2005

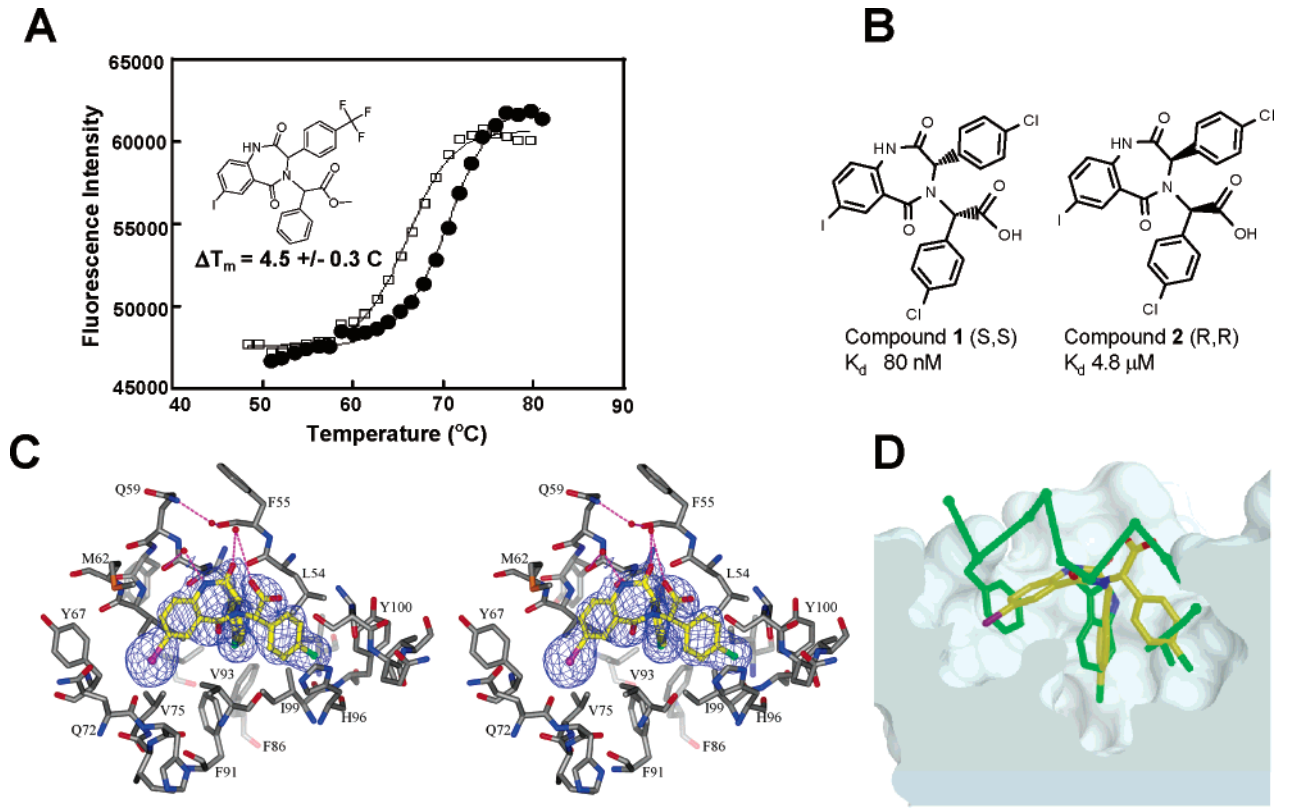

Figure 1. (A) ThermoFluor-generated screening data (□) HDM2 alone, (●) with 50 μM of the compound shown. (B) Enantiomers of an optimized BDP. (C) Stereoview of **1** bound to the p53-binding site of HDM2. Final $2F_o - F_c$ density map contoured at 1.4 σ, with selected HDM2 residues, bound water molecules, and hydrogen bonds. (*D*) Overlay of **1** (yellow) with the 9mer peptide backbone and side chains Phe 19, Trp 23, and Leu 26 (green) in a cut-away molecular surface of HDM2.

affinity and/or size. The structures of HDM2 bound to a 12mer (MPRFMDYWEGLN, data not shown) and a 9mer[20] (RFMDYWEGL, Figure 1D) were very similar to the reported HDM2−p53 15mer complex.[7] The ordered main chain atoms (N, Cα, Cβ, C, O) of corresponding HDM2 residues superimposed with an RMSD of less than 1 Å. The buried surface area decreased with peptide length from 1493 Å^2 to 1207 Å^2 and 1073 Å^2 for the 15mer, 12mer, and 9mer peptides, respectively.

Crystallization trials using BDPs in combination with a series of engineered HDM2 proteins that varied in length lead to the identification of a set of reagents and conditions that consistently yielded diffraction quality crystals. A 2.7 Å crystal structure of **1** bound to HDM2[21] was determined by molecular replacement using the structure of the HDM2−9mer complex as a search model. The salient features of the interaction of **1** with HDM2 are depicted in Figures 1C and 1D. The binding cleft of HDM2 is predominantly hydrophobic,[7] and the inhibitor occupies the same pockets as the peptide side chains Phe 19, Trp 23, and Leu 26. The HDM2 interactions with the inhibitor are, like the bound peptides, largely nonspecific van der Waals contacts.

The compound makes hydrogen bonds to three water molecules (Figure 1C) observed also in other HDM2−BDP cocrystal structures (data not shown), but none to the protein. These water molecules hydrogen bond to a diazepine ring carbonyl that can be removed without substantially affecting the affinity,[19] and to a diazepine ring nitrogen that can be alkylated, again without affecting potency (manuscript in preparation), suggesting that the bound waters do not contribute substantially to the affinity of the BDPs. The antagonist buries roughly 25% less exposed surface area in the p53 binding pocket (804 Å^2) than the area buried by the smallest peptide.

As shown in Figure 1D, the bound conformation of **1** is amphipathic, similar to the p53 peptide. The bound BDP projects pendant groups that orient themselves such that they mimic the position of the hydrophobic side chains on one face of the helical p53 peptide ligand and thus acts as an α-helix mimetic. The discovery of small synthetic molecules that mimic one or more turns of an α-helix has been a significant research challenge.[22−24] To our knowledge, this is the first report of a benzodiazepinedione acting as an α-helix mimetic, suggesting the possible utility of this scaffold in the design of inhibitors of related targets.

Interestingly, the structure of the binding pocket, defined as any HDM2 residue within 4 Å of any atom of **1** (G16, S17, L54, L57, G58, I61, M62, Y67, Q72, F91, V93, H96, I99, Y100), varies little from that observed in the 9mer peptide complex. In contrast, NMR studies reveal global conformational changes in HDM2 on ligand binding.[9,25,26] We have generated cocrystal structures of the *S*,*S* enantiomer and HDM2 from racemic mixtures of similar compounds (data not shown), demonstrating that the HDM2 protein selectively binds the active enantiomer. However, the compounds with IC_{50}s greater than 1 μM in the FP assay failed to produce cocrystals. These data suggest that different tight binding ligands induce a single stable HDM2 conformation.

To determine the activity of HDM2 antagonists on intact cells, JAR choriocarcinoma cells, which overex-

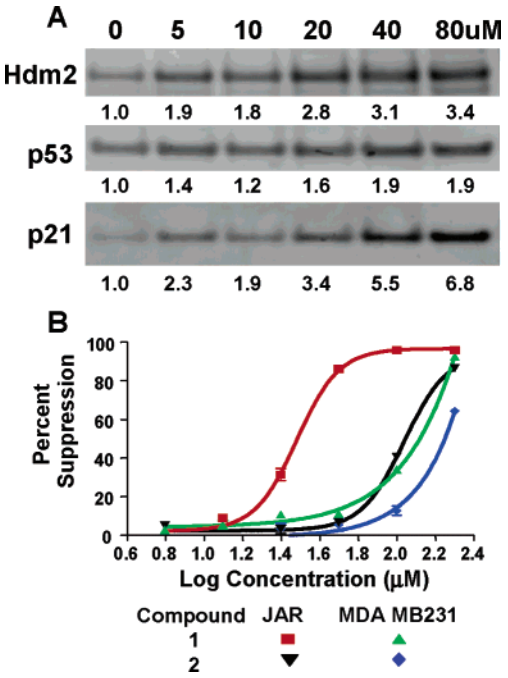

Figure 2. (A) Cellular levels of p53 and p53 target genes in JAR cells after exposure to increasing concentrations of **1**, as determined by Western blot. The value below each band is the ratio of the integrated area to that of the control lane. (B) Dose dependent suppression of proliferation of JAR (wt p53) and MDA-MB-231 (mutant p53) cells after treatment with **1** or **2**.

press p53 and HDM2, were treated with **1.** HDM2 has three roles in association with p53: it inhibits p53-directed transcription, it is itself transcribed, and, as an ubiquitin ligase, it targets p53 for degradation. **1** exhibits an HDM2 antagonist profile in JAR cells: it induces a dose dependent stabilization of p53 protein and an increase in the target genes p21 and HDM2 (Figure 2A). These data support the hypothesis that blocking the binding of HDM2 to p53 renders p53 transcriptionally competent.

Induction of p53 target genes is associated with cell cycle arrest and apoptosis. JAR cells treated with **1** exhibit the expected decrease in proliferation, as measured by incorporation of BrdU, with an IC_{50} of 30 μM (Figure 2B). Cells lacking a functional p53 are predicted to be insensitive to HDM2 antagonists, as was observed for the p53 mutant breast carcinoma cell line MDA MB231 (Figure 2B). The inactive enantiomer, **2**, is not effective at suppressing growth in either p53 wild type or mutant cell lines (Figure 2B). These results strongly support the conclusion that the antiproliferative activities of **1** are mechanistically related to disruption of the HDM2−p53 interaction.

Modulation of protein−protein interactions with small molecule antagonists provides an opportunity for intervention in a wide variety of cellular processes. Success in the development of such antagonists as drugs has so far been limited, although interest in the field is accelerating the pace of the discovery of candidate compounds.[22] Unlike screening assays that monitor the competitive binding of compounds and custom reporter ligands, the ThermoFluor instrument uses generic fluorescent dyes to monitor protein unfolding as a function of temperature, allowing the detection of compound binding to target proteins by measuring the resultant increase in thermal stability. As an affinity-based screen, it requires no specific knowledge of the protein and in principle can be used to discover biologically active compounds that bind to orphan proteins of unknown function. For the HDM2 screen described here, the compounds that produced significant shifts in the melting temperature were tested for site specificity in a fluorescent peptide displacement assay. The identification and optimization of screening hits that antagonize the binding of the HDM2 protein to p53 demonstrates the utility of using ThermoFluor as a screening tool for protein−protein interactions.

The initial structural characterization of the HDM2−p53 peptide complex revealed a binding site that seemed attractive from the viewpoint of small molecule drug discovery.[7] Our results, and those recently reported by another group,[12] provide structural confirmation that synthetic small molecules that have drug-like properties and that compete with p53 for binding to HDM2 can be found and optimized. With respect to the general goal of discovering small molecule antagonists of protein−protein interactions, it is encouraging to discover that multiple chemical classes of small molecule HDM2 ligands bind competitively at the p53 binding site. The BDPs described here induce p53 target genes in cultured tumor cells and decrease proliferation. The HDM2−BDP cocrystal structure should aid in the design of HDM2 antagonists with improved potency and pharmacological properties that may be therapeutically useful in treating tumors by enhancing the activity of endogenous p53.

Supporting Information Available: Synthesis and characterization of **1** and **2**, ThermoFluor peptide binding (Figure S1), FP binding inhibition, crystallographic refinement data, and assay materials and methods. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Levine, A. J. p53, the cellular gatekeeper for growth and division. *Cell* **1997**, *88*, 323−331.
(2) Blaydes, J. P.; Wynford-Thomas, D. The proliferation of normal human fibroblasts is dependent upon negative regulation of p53 function by mdm2. *Oncogene* **1998**, *16*, 3317−3322.
(3) Chene, P.; Fuchs, J.; Bohn, J.; Garcia-Echeverria, C.; Furet, P.; Fabbro, D. A small synthetic peptide, which inhibits the p53-hdm2 interaction, stimulates the p53 pathway in tumour cell lines. *J. Mol. Biol.* **2000**, *299*, 245−253.
(4) Bottger, A.; Bottger, V.; Sparks, A.; Liu, W. L.; Howard, S. F.; Lane, D. P. Design of a synthetic Mdm2-binding mini protein that activates the p53 response in vivo. *Curr. Biol.* **1997**, *7*, 860−869.
(5) Wasylyk, C.; Salvi, R.; Argentini, M.; Dureuil, C.; Delumeau, I.; Abecassis, J.; Debussche, L.; Wasylyk, B. p53 mediated death of cells overexpressing MDM2 by an inhibitor of MDM2 interaction with p53. *Oncogene* **1999**, *18*, 1921−1934.
(6) Wang, H.; Nan, L.; Yu, D.; Agrawal, S.; Zhang, R. Antisense anti-MDM2 oligonucleotides as a novel therapeutic approach to human breast cancer: in vitro and in vivo activities and mechanisms. *Clin. Cancer Res.* **2001**, *7*, 3613−3624.
(7) Kussie, P. H.; Gorina, S.; Marechal, V.; Elenbaas, B.; Moreau, J.; Levine, A. J.; Pavletich, N. P. Structure of the MDM2 oncoprotein bound to the p53 tumor suppressor transactivation domain. *Science* **1996**, *274*, 948−953.
(8) Momand, J.; Jung, D.; Wilczynski, S.; Niland, J. The MDM2 gene amplification database. *Nucleic Acids Res.* **1998**, *26*, 3453−3459.
(9) Stoll, R.; Renner, C.; Hansen, S.; Palme, S.; Klein, C.; Belling, A.; Zeslawski, W.; Kamionka, M.; Rehm, T.; Muhlhahn, P.; Schumacher, R.; Hesse, F.; Kaluza, B.; Voelter, W.; Engh, R. A.; Holak, T. A. Chalcone derivatives antagonize interactions between the human oncoprotein MDM2 and p53. *Biochemistry* **2001**, *40*, 336−344.

(10) Zhao J, W. M., Chen J, Luo A, Wang X, Wu M, Yin D, Liu Z. The initial evaluation of non-peptidic small-molecule HDM2 inhibitors based on p53-HDM2 complex structure. *Cancer Lett.* **2002**, *183*, 69−77.
(11) Galatin, P. S. and Abraham, D. J. A Nonpeptidic Sulfonamide Inhibits the p53-mdm2 interaction and Activates p53-Dependent Transcription in mdm2-Overexpressing Cells. *J. Med. Chem.* **2004**, *47*, 4163−4165.
(12) Vassilev, L. T.; Vu, B. T.; Graves, B.; Carvajal, D.; Podlaski, F.; Filipovic, Z.; Kong, N.; Kammlott, U.; Lukacs, C.; Klein, C.; Fotouhi, N.; Liu, E. A. In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. *Science* **2004**, *303*, 844−848.
(13) Rassokhin, D. N.; Agrafiotis, D. K. Kolmogorov-Smirnov statistic and its applications in library design. *Mol. Graphics Modell.* **2000**, *18*, 370−384.
(14) Agrafiotis, D. K. Stochastic algorithms for maximizing molecular diversity. *J. Chem. Info. Comput. Sci.* **1997**, *37*, 841.
(15) Agrafiotis, D. K.; Lobanov, V. S.; Salemme, F. R. Combinatorial informatics in the post-genomics era. *Nat. Rev. Drug Discovery* **2002**, *1*, 337−346.
(16) Pantoliano, M. W.; Petrella, E. C.; Kwasnoski, J. D.; Lobanov, V. S.; Myslik, J.; Graf, E.; Carver, T.; Asel, E.; Springer, B. A.; Lane, P.; Salemme, F. R. High-density miniaturized thermal shift assays as a general strategy for drug discovery. *J. Biomol. Screen.* **2001**, *6*, 429−440.
(17) Bottger, V.; Bottger, A.; Howard, S. F.; Picksley, S. M.; Chene, P.; Garcia-Echeverria, C.; Hochkeppel, H. K.; Lane, D. P. Identification of novel mdm2 binding peptides by phage display. *Oncogene* **1996**, *13*, 2141−2147.
(18) Bottger, A.; Bottger, V.; Garcia-Echeverria, C.; Chene, P.; Hochkeppel, H. K.; Sampson, W.; Ang, K.; Howard, S. F.; Picksley, S. M.; Lane, D. P. Molecular characterization of the hdm2-p53 interaction. *J. Mol. Biol.* **1997**, *269*, 744−756.
(19) Parks, D. J.; LaFrance, L. V.; Calvo, R. R.; Milkiewicz, K. L.; Gupta, V.; Lattanze, J.; Ramachandren, K.; Carver, T. E.; Petrella, E. C.; Cummings, M. C.; Maguire, D.; Grasberger, B. L.; Lu, T. *Bioorg Med. Chem. Lett*., in press.
(20) PDB code 1T4F.
(21) PDB code 1T4E.
(22) Toogood, P. L. Inhibition of protein−protein association by small molecules: approaches and progress. *J. Med. Chem.* **2002**, *45*, 1543−1558.
(23) Kutzki, O.; Park, H. S.; Ernst, J. T.; Orner, B. P.; Yin, H.; Hamilton, A. D. Development of a Potent Bcl-xL Antagonist Based on −Helix Mimicry. *J. Am. Chem. Soc.* **2002**, *124*, 11838−11839.
(24) Orner, B. P.; Ernst, J. T.; Hamilton, A. D. Toward Proteomimetics: Terphenyl Derivatives as Structural and Functional Mimics of Extended Regions of an Alpha −Helix. *J. Am. Chem. Soc.* **2001**, *123*, 5382−5383.
(25) Schon, O.; Friedler, A.; Bycroft, M.; Freund, S. M.; Fersht, A. R. Molecular mechanism of the interaction between MDM2 and p53. *J. Mol. Biol.* **2002**, *323*, 491−501.
(26) McCoy, M. A.; Gesell, J. J.; Senior, M. M.; Wyss, D. F. Flexible lid to the p53-binding domain of human Mdm2: Implications for p53 regulation. *Proc. Natl. Acad. Sci. U. S. A.* **2003**, *100*, 1645−1648.

JM049137G